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A Novel Retro-Inverso Gonadotropin-Releasing Hormone (GnRH) Immunogen Elicits Antibodies That Neutralize the Activity of Native GnRH

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GnRH vaccines have been successfully used for the inhibition of gonadotropin secretion and gonadal function. As an alternative to native GnRH, retro-inverso (RI) GnRH might be an improved immunogen. The RI peptides are composed of D-amino acids assembled in the reverse order (C to N terminus) in relation to the parent L peptide. These peptides are immunogenic and can produce high titers of antibodies that bind the parent peptide with high affinity and specificity. We show that RI-GnRH peptides conjugated to ovalbumin as well as unconjugated RI-GnRH elicit high titers of anti-GnRH antibodies in rabbits and mice. Antibodies were affinity purified and shown by ELISA to be selective for mammalian GnRH compared with GnRH II and [Gln^8]GnRH. The binding kinetics of antibody-peptide interactions was determined using biosensor technology (BIACORE). The purified anti-GnRH antibodies inhibited GnRH-stimulated signal transduction in COS-1 cells expressing the human GnRH receptor. Immunization of mice with unconjugated and conjugated RI-GnRH peptide, in the absence of complete Freund's adjuvant, produced antisera that cross-reacted with mammalian GnRH. As RI peptides are resistant to cleavage by proteolytic enzymes, they are potentially orally active. The ability of RI-GnRH peptides to produce antibodies to GnRH without conjugation and without Freund's complete adjuvant constitutes a novel vaccine with improved properties of potential application in animal management and sex hormone-dependent cancers. (*Endocrinology* 144: 3262–3269, 2003)

THE DECAPEPTIDE GnRH is synthesized in the neurons of the hypothalamus and released into the portal circulation where it interacts with GnRH receptors on the gonadotrope cells in the anterior pituitary (1). Stimulation of the GnRH receptor is essential for the secretion of LH and FSH, which, in turn, are required for steroidogenesis and gametogenesis (1). Because of this central role in reproduction, GnRH peptide analogs have found therapeutic applications in controlling fertility, cryptorchidism, polycystic ovarian syndrome, leiomyomata, endometriosis, acute intermittent porphyria, and breast and prostate cancer (2, 3) and show promise as new generation contraceptives for men and women.

There is a need for alternative and cost-effective approaches to regulate gonadal activity, particularly in wild and domestic animals and in chronic diseases in man. Immunoneutralization of GnRH by vaccination with synthetic peptides is effective in regulating fertility in animals (4–6) and in the treatment of prostate cancer in men (7), and they have therapeutic potential in sex hormone-dependent neoplasms in woman (8, 9). Peptide-based vaccines have the advantage of being chemically defined and stable as a freeze-dried powder. Peptides do not require large-scale production and are relatively inexpensive. However, a major limitation of peptide vaccines is their relatively low immunogenicity and limited biological half-life (10).

An alternative approach to immunization with native GnRH for the inhibition of gonadotropin secretion and gonadal function is the use of peptidomimetics such as retro-inverso (RI) peptides of GnRH that could serve as vaccines. Developing synthetic vaccines with RI peptides is attractive because it uses stable and chemically defined products with relatively low biological risk and low cost. In RI peptides the residues are aligned in the reverse order to that in the parent peptide and D-amino acids replace the L-amino acids such that the side-chains produce a similar, but non-self, epitope that can be powerful immunogens (10). As the presentation of the amino acid side chains in an RI analog can be very similar to that in the parent peptide, immunization with the analogs may elicit antibodies that cross-react strongly with the parent L-structure (11–13). RI peptides are protease resistant and induce longer lasting immune responses and higher titers of antibodies than do L peptides (10). Their resistance to proteolytic enzymes suggests that they may have oral activity. In addition, antibodies to RI peptides may sometimes have greater affinity than antibodies to classical L peptides and show strong neutralizing activity, as in the case of anti-

Abbreviations: CFA, Complete Freund's adjuvant; G-HCl, guanidinium hydrochloride; IFA, incomplete Freund's adjuvant; IP, inositol phosphates; KSCN, potassium thiocyanate; MAO, maleimide-activated ovalbumin; m-BSA, methylated-BSA; RI, retro-inverso; SPR, surface plasmon resonance; TMB, 3,3′,5,5′-tetramethyl benzidine.

bodies induced to an antigenic site of foot and mouth disease virus (10, 11).

Although GnRH vaccines have been effective in contraception and in a variety of sex hormone-dependent disorders, their peptide nature has necessitated administration by injection along with adjuvant. The development of a potent immunogenic RI-GnRH vaccine with potential oral activity would therefore enhance the therapeutic potential of GnRH immunoneutralization. As a first step in the exploration of this potential, we show that an RI-GnRH peptide is an effective immunogen that induces antibodies that immunoneutralize native GnRH at physiological doses. This demonstrates for the first time that immunization with an RI peptide corresponding to a small peptide hormone produces specific high affinity neutralizing antibodies.

Materials and Methods

GnRH analogs

Mammalian GnRH [pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2 (GnRH)], chicken GnRH I ([Gln^8]GnRH), and GnRH II ([His^5,Trp^7,Tyr^8]GnRH) were prepared by conventional solid phase methodology and purified by preparative C_{18} reverse phase HPLC (University of Cape Town Laboratory).

Peptide immunogen

An RI peptide corresponding to GnRH (RI-GnRH) was synthesized using F-moc technology and purified by HPLC; its mass was verified by mass spectrometry. The sequence of the RI-GnRH peptide was Gly-Pro-Arg-Leu-Gly-Tyr-Ser-Trp-His-Glu-Cys (all D-amino acids), which included the additional cysteine residue at the C terminus for conjugation purposes.

Peptide conjugation

The day before primary injection, 4 mg RI-GnRH were mixed with 2 mg maleimide-activated ovalbumin (MAO; Pierce Chemical Co., Rockford, IL) and incubated for 1 h at room temperature. It was then dialyzed overnight against PBS (10 mM phosphate and 140 mM NaCl, pH 7.4).

Immunization protocol

Two adult female rabbits (Harlan, Leicestershire, UK) were immunized with RI-GnRH (100 μg/rabbit) conjugated with MAO. The primary injections were given sc (1 ml/rabbit) in complete Freund's adjuvant (CFA). These were followed by three booster injections (1 ml/rabbit) in incomplete Freund's adjuvant (IFA) at 2-wk intervals. The last booster injection was given 4 wk later using 200 μg free peptide together with 1 mg methylated BSA (m-BSA). The rabbits were bled 1 wk after each injection.

Two experiments were performed with mice to test whether immunization with RI-GnRH would generate GnRH antibodies, and different methods of immunization with RI-GnRH were tested. In the first experiment, nine male BALB/c mice (Janvier, Toure, France), 9 wk of age, were immunized ip with either 25 μg/mouse RI-GnRH conjugated with MAO (n = 5) or saline buffer (n = 4). The primary injections were given in CFA supplemented with 200 μg m-BSA. These were followed by two booster injections in IFA and m-BSA on d 15 and 45 after the primary injection. The mice were weighed and bled 1 wk after each immunization.

In the second experiment, 4-wk-old BALB/c mice (three of each sex) were immunized with unconjugated RI-GnRH (25 μg/mouse) coinjected with CpG (50 μg/mouse) oligonucleotide (Eurogentech, Brussels, Belgium), and m-BSA (Calbiochem, La Jolla, CA). A control group (two of each sex) received saline buffer together with 50 μg/mouse CpG oligonucleotide (14) supplemented with 200 μg m-BSA. All injections were given in 10% (vol/vol) IFA. Mice were immunized on d 1, 15, and 30. They were bled on d 37 after the initial immunization.

Antibody purification

Serum from rabbits immunized with RI-GnRH peptide was precipitated with saturated ammonium sulfate solution (40%) and dialyzed overnight at 4 C against PBS (pH 7.4). Sepharose 4B beads (1 mg) with activated thiol groups (Pharmacia Biotech, Uppsala, Sweden) were used to couple 4 mM RI-GnRH according to the standard procedures. Immunoglobulins were diluted 15 times to a final concentration of 10 mg/ml and passed through the column (100 μl/min) for 3 h at 4 C. The anti-RI-GnRH antibodies were successively eluted with potassium thiocyanate (3 M KSCN), glycine (2 M; pH 2.8), acetic acid (1% CH_3COOH and 3 M NaCl, pH 2.1), and guanidinium hydrochloride (6 M G-HCl). Two successive fractions of anti-RI-GnRH antibodies were eluted with potassium thiocyanate (KSCN 1 and 2), and one fraction was eluted for each of the other chaotropic agents. All fractions were immediately dialyzed overnight at 4 C against PBS and checked with spectrophotometric analyses.

Enzyme-linked immunoassay

Microtiter plates were coated with RI-GnRH peptide (5 μg/ml) in 100 mM Na_2CO_3 (pH 9.6) and incubated for 1 h at 37 C. After several washes with PBS (pH 7.4), plates were saturated for 1 h with 1% BSA in PBS supplemented with Tween 20 (0.1%, wt/vol) at 37 C. Sera from immunized rabbits and mice and purified rabbit anti-RI-GnRH antibodies at different dilutions (1:500 to 1:3200) were added to the plates and incubated for 1 h at 37 C. Plates were washed several times and allowed to react for 1 h at 37 C with peroxidase-conjugated goat antirabbit or goat antimouse antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h at 37 C. Washed plates were reacted with 3,3′,5,5′-tetramethyl benzidine (TMB) and hydrogen peroxidase as substrate.

Inhibition immunoassay

An inhibition immunoassay was used to test whether native GnRH, [Gln^8]GnRH, or GnRH II could displace purified rabbit anti-RI-GnRH antibodies from binding to fixed RI-GnRH. Nonspecific inhibition was determined with an unrelated L peptide, Val-Arg-Thr-Val-Glu-Asp-Gly-Glu-Cys (V9C), and an unrelated RI peptide (Asp-Ser-Leu-Arg-Asn-Leu-Met-Glu-Cys). Various dilutions of anti-RI-GnRH antibodies were preincubated with increasing concentrations of peptide (3.9–1000 nM) for 1 h at 37 C. The percent inhibition of signal was calculated from the amount of anti-RI-GnRH antibodies that bound RI-GnRH in the presence of increasing peptide concentrations against the amount of anti-RI-GnRH antibodies that bound RI-GnRH in the absence of competing peptide. This immunoassay was also used to test whether native GnRH could displace anti-RI-GnRH antibodies in mouse sera binding to fixed RI-GnRH. Various dilutions of serum samples were preincubated with increasing concentrations of GnRH (3.65 pM to 20 nM) for 1 h at 37 C.

Biosensor experiments

The characterization of binding kinetics of rabbit anti-RI-GnRH antibody binding to RI-GnRH and GnRH was performed with surface plasmon resonance (SPR) technology. The RI-GnRH peptide was fixed on a sensor chip (BIACORE, Uppsala, Sweden) by the standard thiol immobilization protocol using the upgraded BIA 1000 (Pharmacia Biotech). Affinity-purified rabbit anti-RI-GnRH antibodies were injected over the sensor chip at a flow rate of 5 μl/min and a total volume of 100 μl. Anti-RI-GnRH (50 nM) antibodies preincubated with either RI-GnRH (0.01–500 nM) or GnRH (15–5000 nM) for 15 min at room temperature were also injected under the conditions described above. The sensorgrams were recorded and analyzed by BiaEvaluation 3 software (BIACORE).

Transfection of the human GnRH receptor into COS-1 cells and inositol phosphate accumulation assay

The human GnRH receptor gene was cloned into a mammalian expression vector, pcDNA I/AMP (Invitrogen, San Diego, CA) using *Eco*RI

and *Xho*I, and transformed into competent XL-1 blue *Escherichia coli*. Plasmid DNA was extracted with PC100 or PC500 kits (Machery-Nagel, Duren, Germany) from ampicillin-resistant clones and manually sequenced to check for the nucleotide sequence of the human GnRH receptor (Epicentre Technologies, Madison, WI).

COS-1 cells were cultured in DMEM/DMEM (Life Technologies, Inc., Paisley, Scotland, UK), supplemented with 10% fetal calf serum (δ Bioproducts, Kempton Park, South Africa) in a 10% CO_2 incubator at 37 C. The cells were harvested with 0.05% trypsin, seeded into 12-well plates (2×10^5 cells/well) and cultured overnight in DMEM containing 10% fetal calf serum and antibiotics (2 mg/ml streptomycin sulfate, and 4000 U/ml sodium benzyl penicillin). The next day the cells were transiently transfected with the human GnRH receptor, using the DEAE-Dextran method (15).

The transfected COS-1 cells were incubated overnight in 0.5 ml medium 199 (Life Technologies, Inc.) with antibiotics and *myo*-[2-^{3}H]inositol (1 μCi/well; Amersham Pharmacia Biotech, Little Chalfont, UK) as previously described (16). The labeled cells were incubated with various concentrations of GnRH analogs for 1 h at 37 C in the presence of LiCl as described. Aspirating the medium and addition of 10 mM formic acid (1 ml/well) terminated the incubation. Inositol phosphates (IP) were separated from the formic acid extract on DOWEX-1 ion exchange columns and eluted into scintillation liquid (Zinsser Analytical, Frankfurt, Germany), and the radioactivity was counted.

Antiserum inhibition of GnRH-stimulated IP accumulation

Serum from rabbits immunized with RI-GnRH peptide was tested for its ability to inhibit GnRH-stimulated IP accumulation in COS-1 cells transiently transfected with human GnRH receptor. Rabbit serum before immunization was also tested. Serum was diluted 1:250 in buffer containing various concentrations of GnRH (1 and 10 nM). This mixture was incubated for 2 h at 37 C before adding to labeled cells. After 1 h at 37 C the incubation was terminated with formic acid, and IP were separated and counted as described.

The ability of the purified anti-RI-GnRH antibody fractions to inhibit GnRH-, [Gln^8]GnRH-, and GnRH II-stimulated IP production was determined as described above. The effective antibody concentration was calculated by incubating increasing concentrations of purified anti-RI-GnRH antibody fractions (0.1–5 nM) with 0.3 nM GnRH for 2 h at 37 C in buffer. The mixture was added to labeled cells, and the inhibition of IP production was calculated. The amount of purified anti-RI-GnRH antibody needed to inhibit at least 50% of the 0.3 nM GnRH-stimulated IP accumulation was 5 nM. Purified anti-RI-GnRH antibodies were tested for their ability to discriminate among GnRH, [Gln^8]GnRH, and GnRH II. The purified antibody fractions (5 nM) were incubated with 0.3 nM GnRH, 1 nM [Gln^8]GnRH, and 1 nM GnRH II. Stimulating with these concentrations [half-maximal effective concentration (EC_{50})] in the absence of antibody produced similar levels of IP counts. Additionally, purified anti-RI-GnRH antibody (5 nM) was preincubated with increasing concentrations of GnRH (0.1–1000 nM) for 2 h at 37 C in buffer. The mixture was added to labeled cells as described, and the inhibition of IP production was calculated.

Animal treatment

Studies using rabbits and BALB/c mice were conducted with the highest standards of humane animal care in accordance with the Centre National de la Recherche Scientifique Guide for Care and Use of Laboratory Animals. The animals were maintained under controlled temperature and humidity with a 12-h light, 12-h dark cycle and were provided water and standard laboratory diet *ad libitum*.

Data reduction

IP assays were performed in duplicate and four-parameter nonlinear curve fitting (PRISM, GraphPad Software, Inc., San Diego, CA) was used to estimate the peptide concentrations required to stimulate half-maximal IP production (EC_{50}). The formulae for the sigmoidal dose-response curves (unweighted) was defined as Y = bottom + (top − bottom)/(1 + 10^(X − logEC_{50})) (PRISM, version 3.0, GraphPad Software, Inc.). All kinetic analysis was performed with BiaEvaluation 3 (BIACORE) software, using the standard χ^2 statistic test. The mouse anti-RI-GnRH antibody affinities for GnRH were defined as $1/IC_{50} \times$ antibody dilution (IC_{50} is the 50% inhibitory concentration). All statistical analyses were performed with StatView, using a standard correlation program.

Results

Mice immunized with RI-GnRH in the presence or absence of CFA produce anti-RI-GnRH antibodies

In experiment 1 (see *Materials and Methods*) all male mice that were immunized with RI-GnRH peptide conjugated to MAO in CFA (M1-M5) developed anti-RI-GnRH antibodies (Fig. 1) that also bound native GnRH (Fig. 2; by ELISA; see *Materials and Methods*). This indicates that the RI-GnRH antibodies are able to cross-react with the natural L-peptide sequence of GnRH. The ability and specificity of purified RI-GnRH antibodies to interact with GnRH were evaluated with BIACORE, ELISA and tissue culture techniques as shown below.

In experiment 2, mice immunized with unconjugated RI-

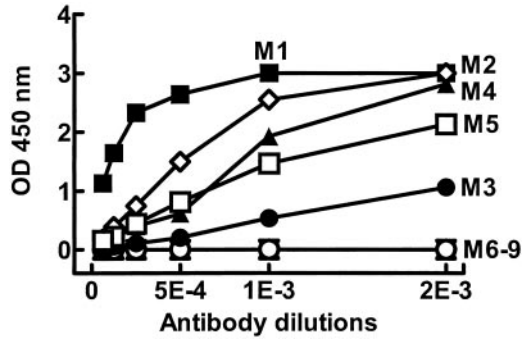

FIG. 1. Titer of anti-RI-GnRH antibodies raised in five male mice (M1–M5) immunized with RI-GnRH conjugated to MAO in CFA in experiment 1. Control mice (M6–M9) received only m-BSA in CFA.

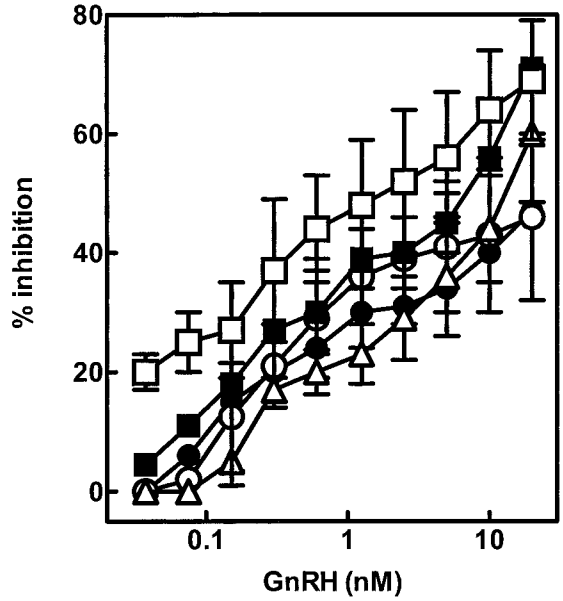

FIG. 2. Serum from male mice immunized with RI-GnRH conjugated to MAO in CFA cross-reacts with native GnRH. RI-GnRH was immobilized on ELISA plates and incubated with immunized serum from male mice (□, M1; ■, M2; Δ, M3; ○, M4; ●, M5) in the presence of increasing concentrations of native GnRH.

GnRH peptide and the mild CpG adjuvant also produced anti-RI-GnRH antibodies (Table 1), showing that RI-GnRH peptide is immunogenic without the need for traumatizing adjuvants.

Rabbit antiserum inhibits GnRH-stimulated IP accumulation

Whole rabbit serum (diluted 1:250) collected after immunization (wk 5) with RI-GnRH peptide could inhibit GnRH-stimulated IP accumulation in COS-1 cells transiently transfected with the human GnRH receptor (Fig. 3). At a concentration of 10 nM GnRH the rabbit serum exhibited 50% inhibition of IP accumulation, and at 1 nM GnRH the antiserum completely inhibited stimulation of IP. Preimmune serum at the same dilution had no effect on GnRH-stimulated IP accumulation. These results demonstrate that serum from rabbits immunized with RI-GnRH inhibit GnRH-stimulated signaling.

Immunized rabbits produced high titer anti-RI-GnRH polyclonal antibodies with high specificity for RI-GnRH

Different dilutions of immunized rabbit serum, affinity-purified anti-RI-GnRH antibodies, and column flow-through were incubated on ELISA plates with immobilized RI-GnRH (see *Materials and Methods*). All purified anti-RI-GnRH antibody fractions bound the RI-GnRH peptide (Fig. 4). Antibody populations eluted by the chaotropic agents acid glycine, CH_3COOH-NaCl, and G-HCl bound the RI-GnRH peptide with lower titer than those eluted with KSCN (KSCN 1), displaying the heterogeneous nature of the raised polyclonal antibodies. The low reactivity of the column flow-through shows that most of the anti-RI-GnRH antibodies were purified from the serum.

Increasing amounts of free RI-GnRH peptide incrementally decreased the amount of anti-RI-GnRH antibodies available to bind the immobilized RI-GnRH peptide (Fig. 5). Binding of all the purified antibody fractions to RI-GnRH was poorly inhibited by a nonrelated L peptide (V9C). As an example, the inhibition of binding by the V9C control peptide of the KSCN 1 antibody fraction to RI-GnRH is shown (Fig. 5). Similarly, anti-RI-GnRH antibody binding was also poorly inhibited with an unrelated RI peptide (data not shown). This shows that these antibodies have high specificity for the RI-GnRH peptide sequence.

TABLE 1. RI-immunized mice produce RI-GnRH antibodies without complete Freund's adjuvant

Mouse	Sex	Titer 1 (d 37)
1	Male	3.00E-05
2	Male	1.00E-03
3	Female	1.00E-02
4	Male	5.00E-04
5	Female	3.00E-05
6	Female	1.30E-04

Male and female mice of proven fertility were immunized with RI-GnRH and CpG without complete Freund's adjuvant on d 1, 15, and 30 and bled on d 37 (experiment 2). Titer is the dilution of serum, containing anti-RI-GnRH antibodies, to yield an absorbance 2.5 times above the blank in ELISA.

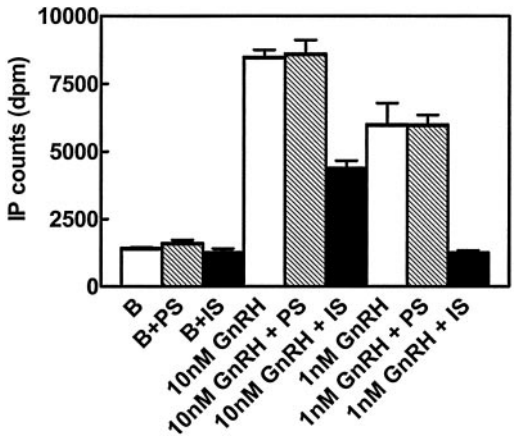

FIG. 3. Anti-RI-GnRH rabbit serum inhibits GnRH-stimulated IP accumulation. Effect of placebo/no serum (□), preimmune rabbit serum (PS; ▨), and anti-RI-GnRH rabbit serum (IS; ■) on basal (B) IP accumulation and GnRH-stimulated (10 and 1 nM) IP accumulation in COS-1 cells transiently transfected with human GnRH receptor.

Immunized rabbits produce antibodies against native GnRH

Native GnRH inhibited purified anti-RI-GnRH antibodies from binding to the immobilized RI peptide on the ELISA plates (Fig. 6), demonstrating that the anti-RI-GnRH antibodies cross-react with the native L-amino acid sequence of GnRH. Of the set of eluted antibody fractions, the KSCN fractions (KSCN 1 and 2) cross-reacted most effectively with native GnRH (Fig. 6) and less so with two other naturally occurring GnRH analogs ([Gln^8]GnRH and GnRH II) that have one ([Gln^8]GnRH) and three ([His^5,Trp^7,Tyr^8]GnRH) amino acid substitutions, respectively. This demonstrates the specificity of anti-RI-GnRH antibodies for GnRH.

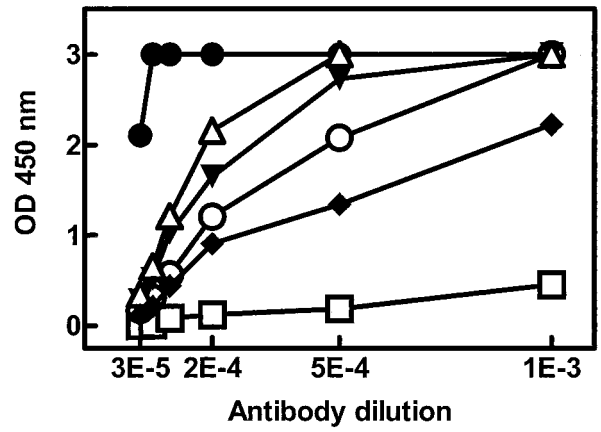

FIG. 4. Titration of affinity-purified fractions of anti-RI-GnRH antibodies from a rabbit immunized with RI-GnRH MOA conjugate. Different dilutions of immunized rabbit serum, purified anti-RI-GnRH antibodies, and column flow-through were incubated on ELISA plates with immobilized RI-GnRH, and the amount of antibody binding was measured as described in *Materials and Methods*. The purified antibody fractions were eluted with KSCN (KSCN 1, ♦), glycine (○), CH_3COOH-NaCl (▼), and G-HCl (Δ). The binding of whole serum (●) and column flow-through (□) is also shown.

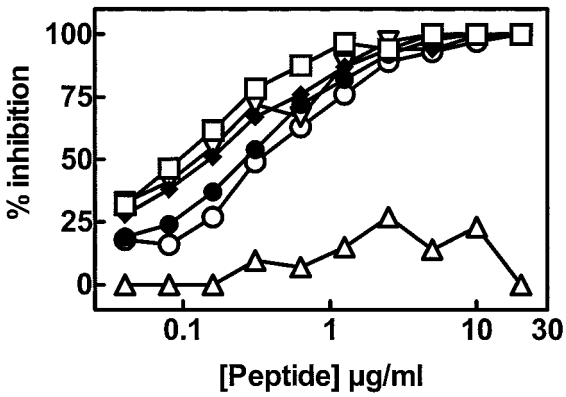

FIG. 5. Purified rabbit anti-RI-GnRH antibodies react with RI-GnRH peptide with high specificity. RI-GnRH was immobilized on ELISA plates and incubated with purified anti-RI-GnRH antibodies. Adding free RI-GnRH peptide to this reaction as described in *Materials and Methods* suppressed the amount of anti-RI-GnRH antibodies that could bind immobilized RI-GnRH. Anti-RI-GnRH antibodies eluted by various chaotropic agents are indicated by *symbols*: ○, KSCN 1; ●, KSCN 2; ♦, glycine; ▽, CH_3COOH-NaCl; and □, G-HCl. The amount of inhibition of KSCN 1 antibody binding to immobilized RI-GnRH by V9C (Δ) is shown as an example of nonspecific interaction with unrelated peptides (see *Materials and Methods*).

Binding kinetics and specificity of two purified anti-RI-GnRH antibody fractions

The binding kinetics of the G-HCl- and KSCN 1-purified antibody fractions were measured with the BIACORE instrument (see *Materials and Methods*). The antibody fraction eluted with G-HCl had more than 40-fold higher affinity for RI-GnRH ($K_d = 1.9 \times 10^{-9}$ M) than the KSCN 1 antibody fraction ($K_d = 7.9 \times 10^{-8}$ M). Additionally, free RI-GnRH could inhibit the G-HCl antibody fraction from binding to the RI-GnRH peptide immobilized on the BIACORE sensor chip more effectively than the KSCN 1 antibody fraction (Fig. 7, *top panel*). This suggests that the G-HCl-eluted antibody fraction is more specific for the RI-GnRH peptide. Conversely, GnRH could inhibit the KSCN 1 antibody fraction from binding to RI-GnRH more effectively than the G-HCl antibody fraction (Fig. 7, *bottom panel*). This indicates that the KSCN 1 antibody fraction has the highest antigenic cross-reactivity with native GnRH. The fact that the presentation of the amino acid side-chains in a RI analog can be very similar to that in the parent peptide (11–13) suggests that the KSCN antibody fractions recognize the native GnRH peptide side-chains with the highest specificity.

Purified anti-RI-GnRH antibodies inhibit GnRH-stimulated IP accumulation

Increasing concentrations of purified anti-RI-GnRH antibodies (KSCN 1 and 2, glycine, and CH_3COOH-NaCl) progressively inhibited 0.3 nM GnRH-stimulated IP accumulation in COS-1 cells transiently transfected with the human GnRH receptor (Fig. 8). The highest available antibody concentration was 5 nM. Consequently, the antibody concentration inhibiting 50% of 0.3 nM GnRH-stimulated IP accumulation was calculated from sigmoidal dose-response curves (PRISM, GraphPad Software, Inc.; see *Materials and Methods*). The EC_{50} for the KSCN 1 fraction was 1.7 nM, that for the KSCN 2 fraction was 1.4 nM, and that for the CH_3COOH-NaCl was 2.2 nM.

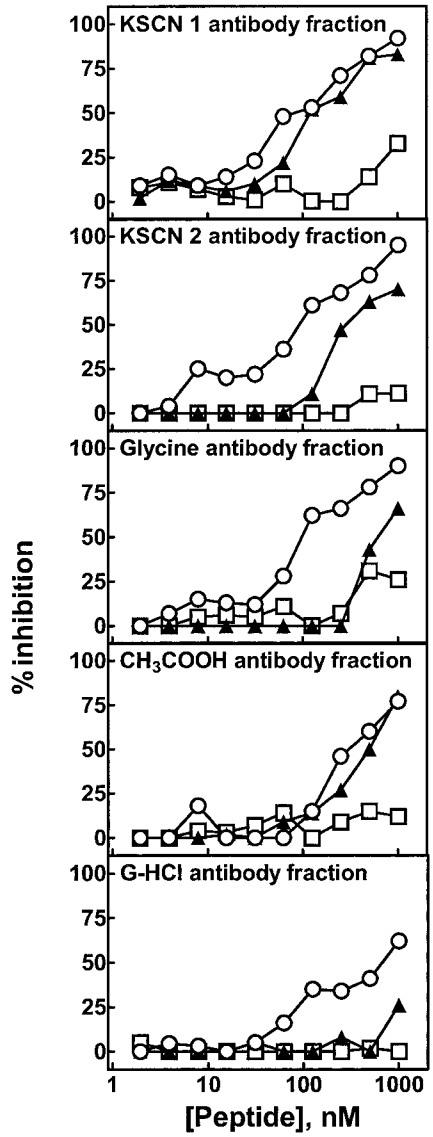

FIG. 6. Purified rabbit anti-RI-GnRH antibody fractions discriminate GnRH from related analogs. RI-GnRH peptide was immobilized on ELISA plates. The binding of purified anti-RI-GnRH antibodies (KSCN 1 and 2, glycine, CH_3COOH-NaCl, and G-HCl) to the fixed RI peptide was suppressed by adding increasing concentrations of free GnRH (○), [Gln^8]GnRH (▲), and GnRH II (□). This inhibition was expressed as a percentage of anti-RI-GnRH antibody binding to immobilized RI-GnRH peptide in the absence of GnRH analogs.

Incubating a fixed concentration (5 nM) of purified anti-RI-GnRH antibodies with varying GnRH concentrations suppressed GnRH-stimulated IP accumulation in COS-1 cells transiently transfected with the human GnRH receptor. The EC_{50} values calculated from sigmoidal dose-response curves of GnRH-stimulated IP accumulation were compared with those for GnRH incubated with anti-RI-GnRH antibodies (Fig. 9). Complete inhibition of GnRH-stimulated IP accumulation was not possible, as only a maximum concentration of 5 nM antibody was available. The EC_{50} values of GnRH-stimulated IP accumulation were effectively sup-

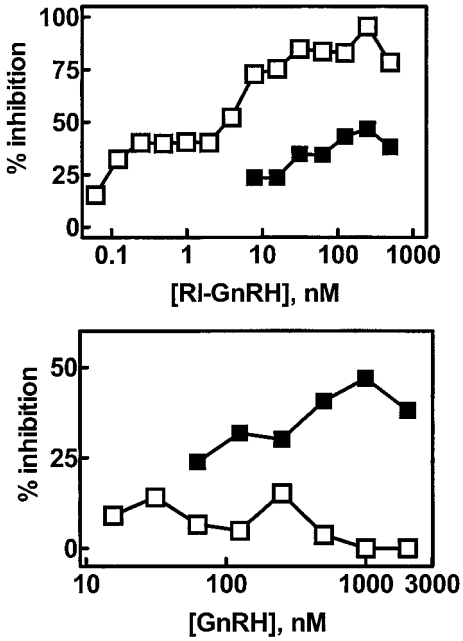

FIG. 7. The ability of purified anti-RI-GnRH antibodies to interact with GnRH and RI-GnRH was measured with BIACORE. RI-GnRH peptide was immobilized to the sensor chip. The amount of anti-RI-GnRH antibody binding (■, KSCN; □, G-HCl) to RI-GnRH peptide was measured in resonance units (RU) and was suppressed by adding increasing concentrations of RI-GnRH peptide (*top panel*) or GnRH (*bottom panel*). This inhibition was expressed as a percentage of anti-RI-GnRH antibody binding to immobilized RI-GnRH peptide in the absence of free RI-GnRH peptide or GnRH.

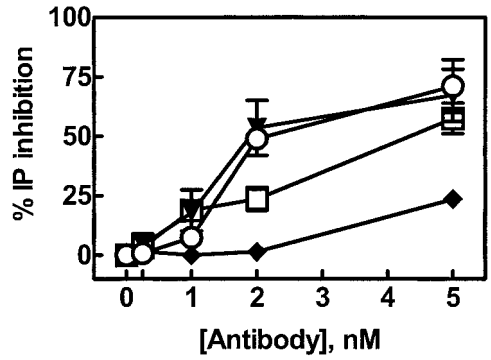

FIG. 8. Effect of increasing concentrations of purified rabbit anti-RI-GnRH antibody fractions on GnRH-stimulated IP accumulation. A fixed concentration of GnRH (0.3 nM) was preincubated with varying concentrations of rabbit anti-RI-GnRH antibody from different eluted fractions. The concentration of anti-RI-GnRH antibody needed to inhibit 50% of the GnRH-stimulated IP accumulation in COS-1 cells transiently transfected with the human GnRH receptor was determined (sigmoidal curve, PRISM, GraphPad Software, Inc.) for fractions KSCN 1 (1.7 nM; ○), KSCN 2 (1.4 nM; ▼), glycine (2.2 nM; □), and CH_3COOH-NaCl (not calculated; ◆).

pressed by the KSCN 1-eluted (14.4-fold), KSCN 2-eluted (6.0-fold), and glycine-eluted (4.4-fold) antibody fractions (Fig. 9). The KSCN 1 antibody fraction could inhibit 0.1 nM GnRH-stimulated IP accumulation by more than 90%. The concentration of GnRH in the hypothalamic-hypophyseal portal system is similar to this dose.

The ability of the different purified anti-RI-GnRH anti-

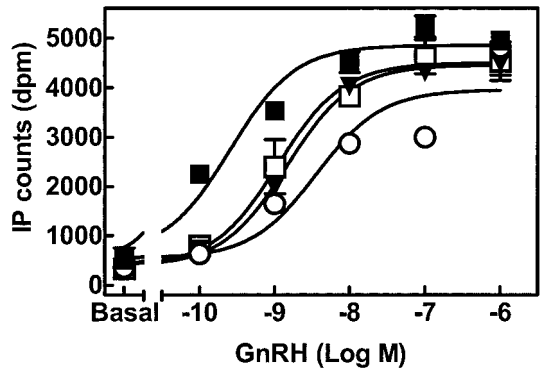

FIG. 9. Purified rabbit anti-RI-GnRH antibodies suppress increasing concentrations of GnRH-stimulated IP accumulation. Dose-response curves of GnRH-stimulated IP accumulation were measured in COS-1 cells transiently transfected with the human GnRH receptor (sigmoidal dose-response curve, PRISM, GraphPad Software, Inc.). The EC_{50} of GnRH (0.25 nM; ■) was suppressed when 5 nM anti-RI-GnRH antibody from fractions KSCN 1 (3.6 nM; ○), KSCN 2 (1.5 nM; ▼), and glycine (1.1 nM; □) were preincubated with varying concentrations of GnRH.

body fractions to inhibit GnRH-, [Gln^8]GnRH-, and GnRH II-stimulated IP accumulation was compared in COS-1 cells transiently transfected with the human GnRH receptor (Fig. 10). The KSCN 1-, KSCN 2-, and glycine-eluted antibody fractions inhibited GnRH-stimulated IP production more than that of GnRH II (compare with Fig. 6). Moreover, the KSCN-eluted antibody fractions inhibited GnRH-stimulated IP accumulation better than the glycine-eluted antibody fraction. The CH_3COOH-NaCl- and G-HCl-eluted antibody fractions did not inhibit any of the GnRH analog-stimulated IP production (data not shown). This suggests that the KSCN-eluted antibody fraction cross-reacted the most with native GnRH and is consistent with the results of the inhibition ELISA (Fig. 6) and BIACORE (Fig. 7) experiments. Additionally, the RI-GnRH peptide alone could not stimulate or inhibit GnRH-stimulated IP accumulation in COS-1 cells transiently transfected with the human GnRH receptor (data not shown). This shows that the RI-GnRH peptide has no effect on GnRH receptor function, other than acting as an immunogen producing neutralizing GnRH-specific antibodies.

Discussion

This study has demonstrated that an RI peptide can be used as a synthetic GnRH vaccine. Immunization of rabbits and mice with an RI-GnRH peptide elicits the production of polyclonal anti-RI-GnRH antibodies that cross-react with the natural L-amino acid peptide, GnRH, as measured with ELISA and SPR. Sera containing anti-RI-GnRH antibodies were able to effectively immunoneutralize GnRH-stimulated IP accumulation in COS-1 cells expressing the human GnRH receptor.

Although RI peptides have previously been reported to induce the production of antibodies that immunoneutralize the infectivity of viruses containing the shell of native L-amino acid proteins (11, 13) and large peptides (12), RI peptides have not yet been employed to immunoneutralize small biologically active peptides such as GnRH. As the N and C

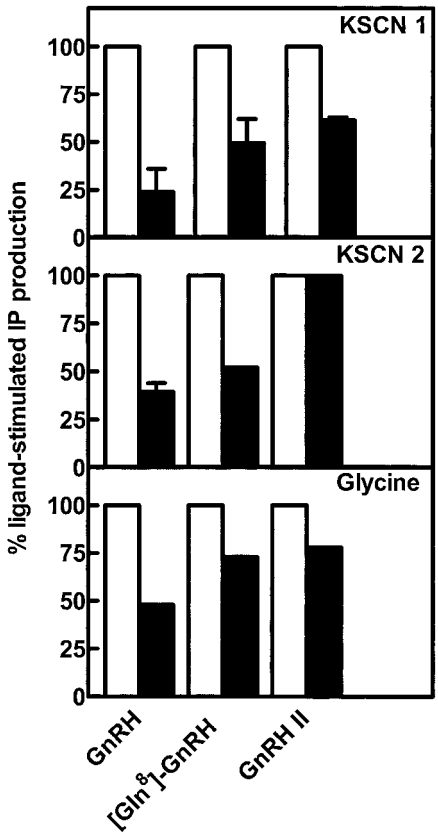

FIG. 10. Purified rabbit anti-RI-GnRH antibodies selectively inhibits GnRH-stimulated IP accumulation. COS-1 cells transfected with the human GnRH receptor were incubated separately with 0.3 nM GnRH, 1 nM [Gln^8]GnRH, and 1 nM GnRH II, which were preincubated with 5 nM anti-RI-GnRH antibody fractions KSCN 1, KSCN 2, and glycine (■). The effect of the antibodies on ligand-stimulated IP accumulation was calculated as the percent inhibition of respective ligand-stimulated IP accumulation in the absence of antibody (□).

termini (pGlu and Gly·NH_2), which are important for binding of GnRH to its cognate receptor (17), cannot be simulated in RI-GnRH [NH_2-CH(C_2H_4COOH)-CO- and NH_2-CH-CO-], it was not predictable that antibodies raised against RI-GnRH would immunoneutralize the native peptide. Clearly the antisera were not against the N and C termini of RI-GnRH, as these would not recognize native GnRH. As the antibodies cross-react less with [Gln^8]GnRH and GnRH II, which differ in structure in the central region (Gln^8 and His^5, Trp^7, Tyr^8, respectively), it appears that the antibodies are directed at this region. Their ability to immunoneutralize GnRH suggests that binding the central region sterically impairs access of the N and C termini to the receptor.

Antibodies from whole rabbit serum were precipitated, and the anti-RI-GnRH antibodies were affinity purified and characterized with ELISA and the BIACORE 1000 system (18). The anti-RI-GnRH antibody fractions had differing affinities and specificities for RI-GnRH and mammalian GnRH. The antibodies with the highest affinity for RI-GnRH peptide had, as anticipated, the lowest cross-reactivity with GnRH, whereas those with lower affinity for RI-GnRH cross-reacted well with GnRH. Nevertheless, the lower affinity antibodies were of high titer and relatively selective for mammalian GnRH compared with [Gln^8]GnRH and GnRH II. These antibodies are probably the main contributors to the inhibition of GnRH-stimulated signal production in COS cells expressing the human GnRH receptor. The specificity of the antibodies against mammalian GnRH was encouraging, as most vertebrate species have variant forms of GnRH, which may have different physiological functions (19, 20). For example, rhesus and cynomolgus monkeys and man have both GnRH and GnRH II, of which GnRH II is highly expressed in extrahypothalamic brain areas (21, 22) and is suggested to have a neuromodulator role (20, 23). To specifically inhibit the reproductive system, it is desirable that antibodies raised against RI-GnRH do not cross-react with GnRH II, as has been demonstrated by these studies.

The study demonstrated that the synthetic RI-GnRH peptide elicits high titers of anti-GnRH antibodies, which immunoneutralize mammalian GnRH. The studies in male and female mice established that antibodies to RI-GnRH also bound the native GnRH peptide. Observations on pregnancy outcome of the matings of immunized male or female mice indicated an inhibition of fertility (data not shown). However, analysis of plasma pituitary and gonadal hormones was not undertaken. As such, the study represents a proof of concept and a point of departure for detailed studies on *in vivo* effects of RI-GnRH peptide antisera. These RI-GnRH immunogens have a number of potential advantages over previously published GnRH vaccination approaches that employ GnRH conjugated to carrier proteins and adjuvants that seriously compromise recipients. Firstly, immunization with RI-GnRH does not require conjugation to an immunogenic carrier protein, as the RI-GnRH is apparently not recognized as self, but is sufficiently similar to GnRH to produce GnRH-immunoneutralizing antibodies. Secondly, the RI peptides tend to produce higher titers (10, 11). Thirdly, when administered with the oligonucleotide CpG, antibodies are produced without the need for the traumatizing CFA. Fourthly, as D-amino acid peptides are resistant to proteases (10), it is possible that the RI-GnRH immunogen will be active as an oral vaccine. As an alternative, it is feasible that conjugation to bile salts, attenuated toxins (*e.g.* pertussis and cholera), and actively absorbed vitamins may facilitate absorption across the gastrointestinal tract, thereby eliciting a specific IgG response.

GnRH vaccines are thought to be the most practical for use in companion animal contraception (24), in animal husbandry (5), and in controlling wildlife populations (6). Although GnRH vaccines are also potential contraceptive agents in humans (25), concerns about efficacy, reversibility, and the need to supplement sex hormones would have to be addressed. The most credible application in humans would be in the treatment of chronic and life-threatening sex hormone-dependent neoplasms (7). This is especially pertinent in developing countries where GnRH analog treatment is not economically viable.

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